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Circannual pupation timing is not correlated with circadian period in the varied carpet beetle *Anthrenus verbasci*

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Anthrenus verbasci (Insecta, Coleoptera, Dermestidae) shows a circannual rhythm in pupation and a circadian rhythm in adult locomotor activity. The period length of the circannual rhythm will be correlated with the length of a circadian period if oscillations of these two rhythms share a common genetic background. We examined the relationship between the two rhythms and found no correlation between the pupation time determined by the circannual rhythm and the circadian period of the adult activity rhythm. This result suggests that the mechanism that produces the circannual rhythm of *A. verbasci* is not affected by the period of circadian rhythm.

Keywords: circadian rhythm; circannual rhythm; locomotor activity

Introduction

Circannual rhythms are endogenous biological rhythms that regulate seasonal development and behavior in various species (Gwinner 1986). It has been suggested that the circannual rhythm is derived from an endogenous clock with a period of about a year, i.e. a circannual clock, which is independent of the circadian clock (Gwinner 1986; Miyazaki et al. 2012). In the European starling *Sturnus vulgaris*, Gwinner (1973) observed a weak positive correlation between the circadian period of activity rhythm and the circannual period of molt rhythm under constant dim light. He presumed that this correlation resulted from the action of common endogenous factors affecting both rhythms (Gwinner 1981, 1986).

In circadian rhythm mutants of *Drosophila melanogaster* and the melon fly *Bactrocera cucurbitae*, the circadian period is correlated with development time and the period of ultradian rhythms that are related to control of the courtship song (Kyriacou and Hall 1980; Kyriacou et al. 1990; Shimizu et al. 1997; Alt et al. 1998; Miyatake and Kanmiya 2004). These results indicate that circadian clock genes have pleiotropic effects on the development rate and period of other endogenous biological rhythms. We regard these effects as candidate causal factors affecting both circadian and circannual rhythms, presumed by Gwinner (1981, 1986).

The varied carpet beetle *Anthrenus verbasci* regulates the timing of pupation by a circannual rhythm. This rhythm persists with a period of approximately 37 weeks and is entrained by photoperiodic cues to an environmental cycle (Nisimura and Numata 2001). Under constant conditions, the pupation time of *A. verbasci* exhibits some individual variation within a given circannual cycle. This variation can be attributed to individual differences in the period of circannual rhythm (Miyazaki et al. 2005). In the present study, we examined the possibility that the pleiotropic effects of circadian clock genes influence the periodicity of the circannual clock of *A. verbasci*. However, we could not measure the circadian period of *A. verbasci* larvae because of their immobility, but adults showed a clear rhythm in locomotion. We, therefore, recorded the pupation time of *A. verbasci* under a constant photoperiod of 12 h of light and 12 h of darkness (LD 12:12), or under constant light (LL), and then recorded the locomotor activity of adults. We traced the relationship between pupation time and the circadian period of adults to examine the genetic correlation between circadian and circannual rhythms.

Materials and methods

Adults of *A. verbasci* were collected in Minoh City, Japan (34.8°N, 135.5°E), in May 2009. These insects and their eggs were maintained under LD 16:8 at $25 \pm 1^\circ\text{C}$, and larvae within 1 week after hatching were transferred to LD 12:12 at $20 \pm 1^\circ\text{C}$ and 66% relative humidity. Dried bonito powder and dried yeast were provided as larval food (Nisimura and Numata 2001). The photoperiod was produced using white fluorescent lamps (FL 15 W; Panasonic, Kadoma, Japan) and timers, and light intensity during the photophase was approximately 0.5 Wm^{-2} .

Larvae reared under LD 12:12 were either transferred to LL at $20 \pm 1^\circ\text{C}$, 8 weeks after the start of the experiment, or maintained continuously under LD 12:12. Under both conditions, *A. verbasci* showed a clear circannual rhythm in pupation (Miyazaki and Numata 2010). Pupae were classified into first and second pupation groups based on whether pupation occurred in the first or second cycle of the circannual rhythm. In the first pupation group, some of the pupae were transferred from LD 12:12 to LL, or vice versa, to examine whether the photoperiod during the pupal and adult stages is needed for the expression of circadian rhythm in adults. In the second pupation group, all pupae were maintained under LD 12:12.

Adults emerged 14–16 days after pupation but remained inactive in the last larval exuvia for 7–15 days. Activity was recorded using adults within 10 days of egression from the exuvia (22–41 days after pupation). An adult beetle was retained in an activity chamber (33 × 33 × 4 mm), and water was provided by placing wet cotton wool. An infrared beam (EE SPW-321; Omron, Kyoto, Japan) was passed across each chamber, and the number of beam interruptions was recorded at 6-min intervals on a personal computer. Locomotor activity was recorded for 10 days under LL (0.35–2.8 Wm⁻²) at 20 ± 1°C. Rhythmicity and circadian periods were evaluated by a chi-square periodogram (Sokolove and Bushell 1978) and by visual inspection.

The chi-square test was used to examine whether the pupation groups and light conditions to which insects had been exposed before activity recording affect the proportions of adults showing a circadian rhythm. The Steel-Dwass test for nonparametric multiple comparison was used to examine whether the pupation groups and light conditions before activity recording affect the circadian periods of adults. The relationship between pupation time and the circadian period of adults was analyzed by calculating the Kendall's rank correlation coefficient.

Results

Circannual pupation rhythm

Under LD 12:12, 489 larvae pupated 20–29 weeks after hatching, and the median larval duration of this first pupation group was 24 weeks. Four larvae pupated 60–66 weeks after hatching, and the median larval duration of this second pupation group was 63.5 weeks. The interval between the two medians was 39.5 weeks (Figure 1a). Under LL, pupation of the first group occurred 16–35 weeks after hatching, and the median larval duration was 26 weeks. Thirty-one larvae pupated 45–76 weeks after hatching, and the median larval duration of this second pupation group was 59 weeks. The interval between the two medians was 33 weeks (Figure 2a).

Circadian activity rhythm

A clear circadian activity rhythm persisted under LL in 220 out of 299 adults (Table 1, Figure 3). The pupation groups and light conditions to which insects had been exposed before activity recording did not significantly affect the proportion of adults showing a

clear rhythm (chi-square test, $df = 4$, $\chi^2 = 6.31$, $P > 0.05$) and the circadian period (Steel-Dwass test, $P > 0.05$).

Relationship between larval duration and free-running circadian period

Individual variation was observed both in the larval duration and in the circadian free-running period, even among individuals that pupated within a same circannual cycle. However, there was no significant correlation between larval duration and circadian period, regardless of pupation group and light conditions in larval, pupal, and inactive adult stages ($P > 0.05$, Figures 1b–d and 2b–d).

Discussion

Although *A. verbasci* adults show a clear diurnal activity pattern outdoors (e.g. Kato 1941), to date, there has been no report of adult activity under constant conditions. The present study revealed that adult locomotor activity is controlled by a circadian rhythm. This rhythm persisted in many adults under LL, as has been shown in many other coleopterans (see Saunders 2002), even in the absence of a photoperiod during the pupal and adult stages. We examined whether the circadian period of this rhythm is correlated with larval duration, which is determined by circannual rhythm. After the larvae were transferred to LL, their circadian rhythm should have been free-running, because the circadian activity rhythm of many adults free-ran under LL. If a direct causal relationship exists between circadian and circannual rhythms or if a common endogenous factor determines the periods of both rhythms, the circadian period of the activity rhythm will be positively correlated with the period of the circannual rhythm, as shown in *S. vulgaris* (Gwinner 1973). However, there was no correlation in *A. verbasci* between the circadian period of adult activity rhythm and larval duration. Kenagy (1981) also showed no correlation under LL between the circadian period of locomotor activity and the circannual period of the testicular cycle in four males of the antelope ground squirrel *Ammospermophilus leucurus*.

In *D. melanogaster* and *B. cucurbitae*, a circadian clock gene(s) has pleiotropic effects on the circadian rhythm, development rate, and ultradian rhythm; therefore, the circadian period is correlated both with development time and with the period of ultradian rhythms in the courtship song (Kyriacou and Hall 1980; Kyriacou et al. 1990; Shimizu et al. 1997; Alt et al. 1998; Miyatake and Kanmiya 2004). In the adzuki bean

beetle *Callasobruchus chinensi*, however, there is no correlation between circadian period and development time among different strains (Harano and Miyatake 2011). Harano and Miyatake (2011) reported that the genes or mechanisms underlying the development of beetles may be different from those underlying the development of flies. This expectation may apply to larval development in *A. verbasci*. Whether an *A. verbasci* larva pupates in the first, second, or later circannual cycle depends on the development rate, because only sufficiently grown larvae can pupate within a given circannual cycle (Miyazaki et al. 2009). In the present study, there was no significant difference in circadian periods between the first and second pupation groups of *A. verbasci*. This finding is consistent with the results for *C. chinensi*. In addition, the difference in the circadian periods of *A. verbasci* did not reflect individual variation in pupation time even in the same circannual cycle. These results suggest that the oscillation of circannual rhythm in *A. verbasci* is independent of the period of circadian rhythm.

However, we cannot exclude the possibility that a circadian period is different between the larval and adult stages and that different circadian oscillators are involved in the circannual pupation rhythm and in adult locomotor activity. Recently Takekata et al. (2012) showed that knockdown of a clock gene *period* by RNA interference disrupts the circadian rhythm but not the circatidal rhythm in the mangrove cricket *Apteronomobius asahinai*. A similar molecular approach would be helpful to clarify directly the relationship between circadian and circannual rhythms in *A. verbasci*.

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Table 1. The proportion of adults showing a circadian locomotor rhythm and the circadian period in *Anthrenus verbasci*.

Pupation group	Light conditions (20°C) ¹		<i>n</i>	Clear rhythm (%) ²	Circadian period (h, median) ³
	Larva	Pupa/inactive adult			
1st	LD	LD	62	83.9	23.8
	LD	LL	47	66.0	23.9
	LL	LD	92	75.0	23.7
	LL	LL	67	67.2	24.0
2nd	LD	LD	3	100.0 ⁴	24.6 ⁴
	LL	LD	28	71.4	24.35
Total			299	73.6	23.85

¹ LD, 12 h of light and 12 h of darkness; LL, constant light.

² There were no significant differences among the five experimental groups (chi-square test, $df = 4$, $\chi^2 = 6.31$, $P > 0.05$).

³ There were no significant differences among the five experimental groups (Steel-Dwass test, $P > 0.05$).

⁴ These values were excluded from statistical tests because of the small sample size.

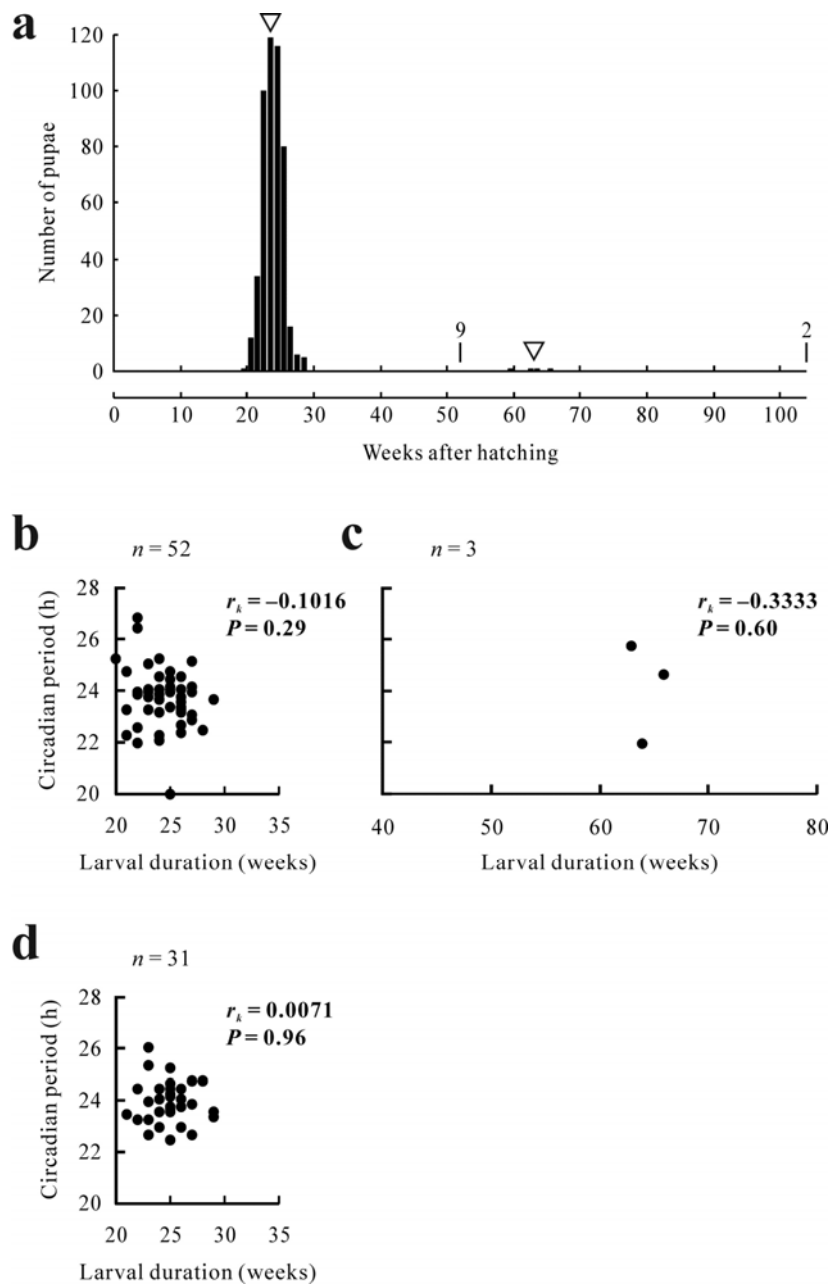


Figure 1. Circannual pupation rhythm under LD 12:12 at 20°C (a) and correlation between larval duration and the circadian period of adult activity under LL at 20°C (b–d) in *Anthrenus verbasci*. The two numbers above the vertical lines indicate the number of larvae remaining after 52 and 104 weeks, respectively, and the two triangles indicate the median larval duration of the first and second pupation groups, respectively (a). Light conditions during pupal and inactive adult stages before activity recording are LD 12:12 (b, first pupation group; c, second pupation group) or LL (d, first pupation group). Kendall's rank correlation coefficient and its probability are indicated as r_k and P , respectively (b–d).

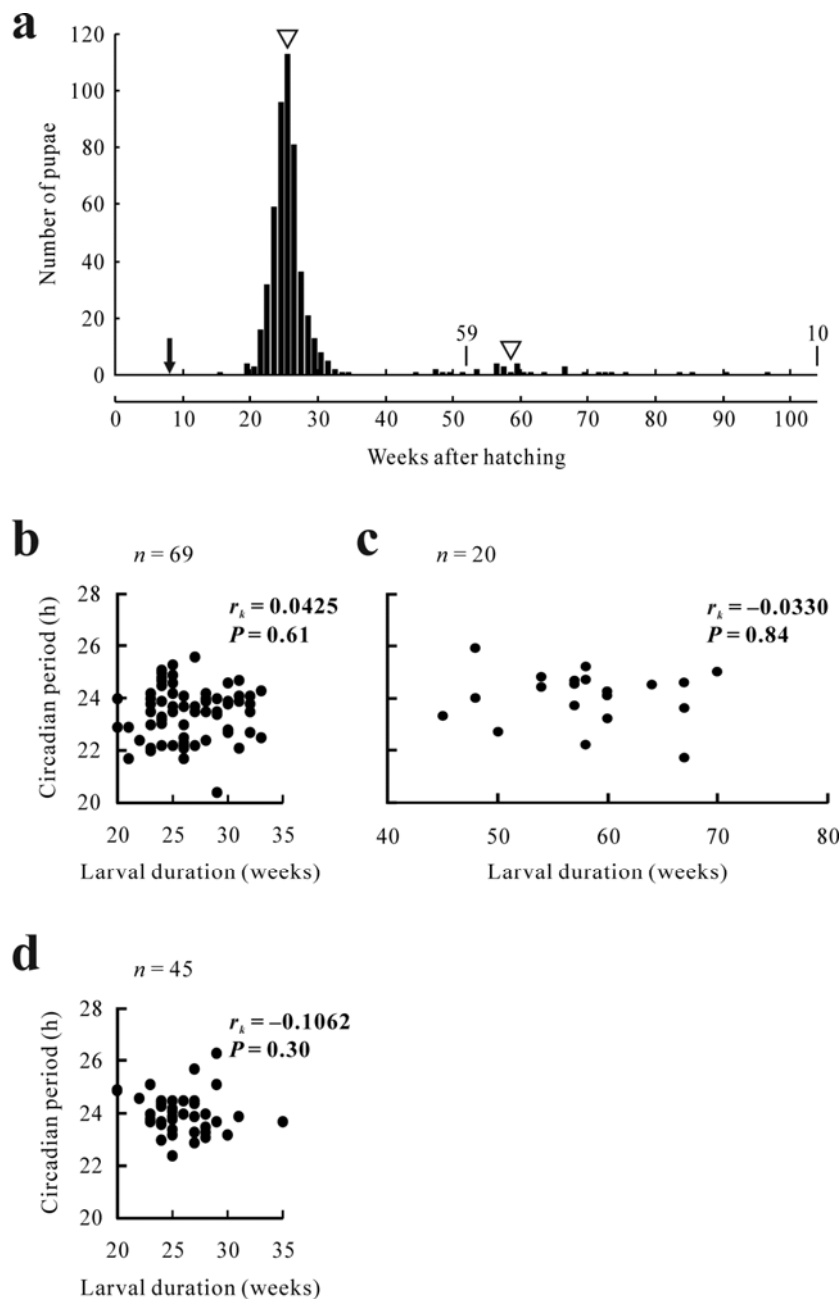


Figure 2. Circannual pupation rhythm under LL at 20°C (a) and correlation between larval duration and the circadian period of adult activity under LL at 20°C (b–d) in *Anthrenus verbasci*. An arrow shows the transfer from LD 12:12 to LL, 8 weeks after hatching; the two numbers above the vertical lines indicate the number of larvae remaining after 52 and 104 weeks, respectively; and the two triangles indicate the median larval duration of the first and second pupation groups, respectively (a). Light conditions during pupal and inactive adult stages before activity recording are LD 12:12 (b, first pupation group; c, second pupation group) or LL (d, first pupation group). Kendall's rank correlation coefficient and its probability are indicated as r_k and P , respectively (b–d).

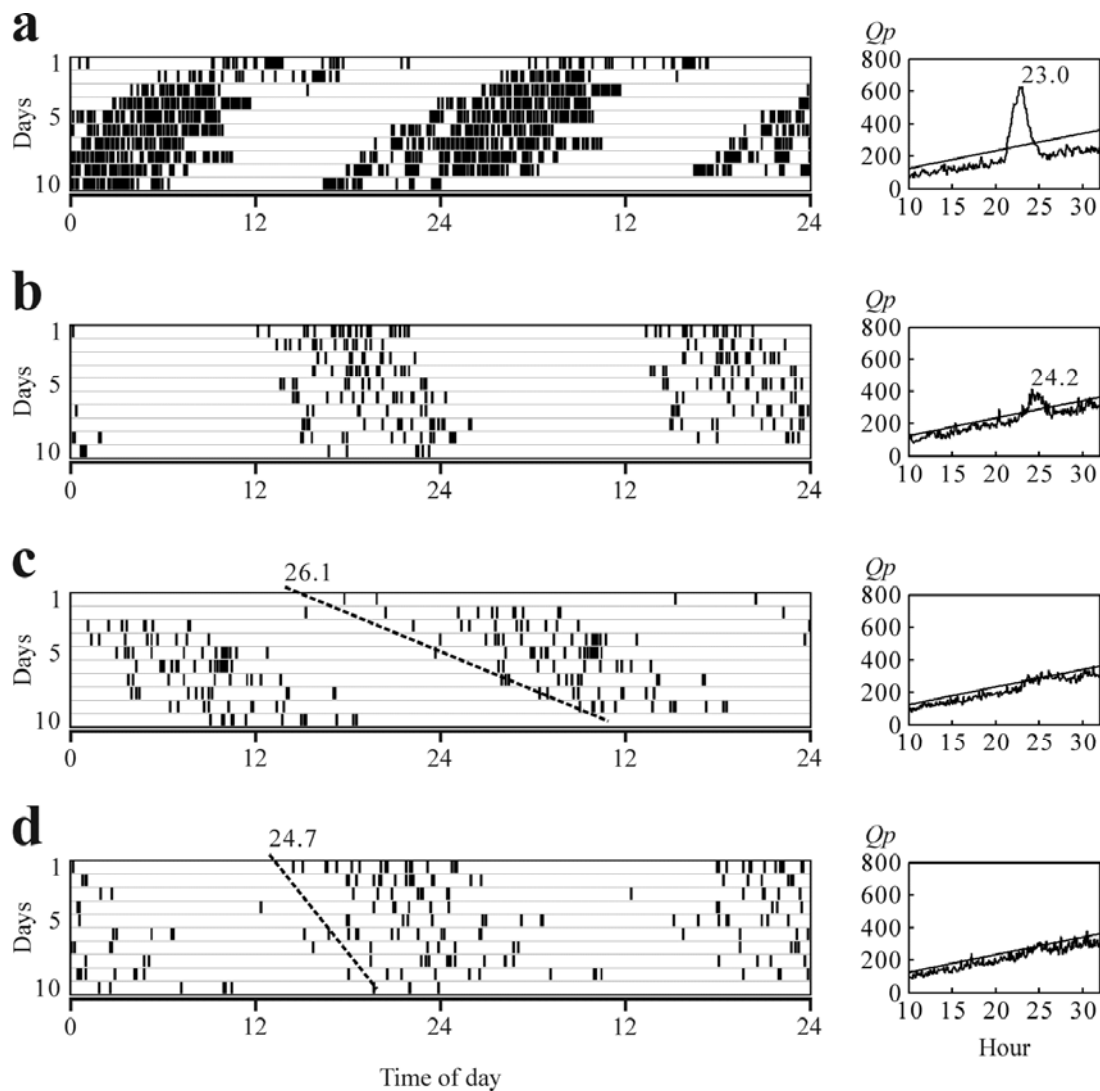


Figure 3. Representative double-plotted actograms of a clear circadian activity rhythm in adults of *Anthrenus verbasci* under constant light at 20°C for 10 days (left) and their chi-square periodogram analyses (right). Rhythmicity and circadian periods were evaluated by a chi-square periodogram (a, b) or by visual inspection (c, d). The free-running periods are shown in the periodograms (a, b) or the actograms (c, d). The oblique lines in the periodograms indicate a significance level of $P = 0.05$. Qp is the statistic value (ratio of variances) of the chi-square periodogram (Sokolove and Bushell 1978).